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Title

Effect of storage temperature, nitrogen gassing and sperm concentration on the *in vitro* semen quality *and in vivo* fertility of liquid bull semen stored in INRA96

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Abstract

The aim of this study was to assess the effect of storage temperature, nitrogen (N_2) gassing and sperm concentration on *in vitro* characteristics and calving rate (CR) following artificial insemination (AI) of liquid bull semen stored in INRA96. In Experiment 1 the effect of liquid bull semen diluted in either N_2 bubbled or non-bubbled INRA96 at a concentration of 5 x 10^6 sperm per 0.25 mL insemination dose and stored at 4 or 15 ^oC was assessed subjectively for total and progressive motility on Days 0, 1, 2, 3 and 4 post collection. In Experiment 2a, the effect of stored liquid semen at three sperm concentrations (3, 4 or 5 x 10^6 sperm per 0.25 mL insemination dose) on total and progressive motility was assessed subjectively on Days 0, 1 and 2 post collection. In Experiment 2b, the field fertility of liquid semen stored at ambient temperature at a concentration of 3, 4 or 5 x 10^6 sperm per 0.25 mL dose and inseminated on Days 1 or 2 post collection was assessed in comparison to frozen-thawed semen (total of n = 5,742). In Experiment 1, total and progressive motility decreased with increased duration of storage ($P < 0.01$); however, there was no effect of N_2 bubbling on motility on Days 0, 1, 2, 3 and 4 of storage. There was an effect of temperature on total and progressive motility, regardless of treatment, as semen stored at 15 ^oC recorded higher motility values than semen stored at 5 ^oC ($P < 0.01$). In Experiment 2a, there was no effect of sperm concentration on total or progressive motility on Days 0, 1 or 2 of storage. There was a linear decrease in motility with increased duration of storage ($P < 0.01$); however, there was no sperm concentration by day interaction. In Experiment 2b, there was an effect of sperm concentration on CR ($P < 0.01$); semen diluted to 3 and 4 x 10^6 sperm per dose resulted in a lower CR after 2 days of storage (41.1 and 44.7%, respectively) in comparison to frozen-thawed semen (55.2%) but did not differ to CR of semen diluted to 5 x 10^6 sperm per dose on Day 2 of storage. There was an effect of parity, fertility sub-index and days in milk (DIM) at AI on CR ($P < 0.01$). In conclusion, N_2 bubbling and sperm concentration had no effect on *in*

vitro sperm motility of liquid semen, but this study demonstrated a reduction in CR on Day 2 of storage at lower sperm concentrations in comparison to frozen-thawed semen.

1. Introduction

Seasonal grass-based dairy production systems depend on compact breeding during mid-April to early June in the Northern Hemisphere, in order to coincide milk production with grass growth [1]. Artificial insemination (AI) is the single most important technique devised to facilitate the genetic improvement of animals [2, 3] and currently, within the Irish dairy industry, 95% of AI is conducted using frozen-thawed semen, with liquid (i.e., fresh, non-cryopreserved) semen accounting for only 5% of annual inseminations [4]. However, the use of liquid semen increases to approximately 25% during the peak breeding season in order to accommodate the large demand [5]. Although liquid bull semen has traditionally been stored at ambient temperature in the egg-yolk based diluent, Caprogen, Murphy et al. [6] indicated that bull semen stored in INRA96 (a commercially available milk-based diluent) had a comparable calving rate (CR) to Caprogen but was more convenient for the busy working schedule of an AI centre as it could be used directly off the shelf.

In Ireland a typical liquid semen dose contains 5 x 10^6 sperm, irrespective of its usage day [7], in comparison to 15 x 10^6 sperm for a typical frozen-thawed semen dose [8]. Thus, liquid semen processing yields more doses per ejaculate, thereby facilitating the greater utilisation of genetically superior sires. This is particularly beneficial for young genomically-selected sires as these sires are in high demand but produce lower semen volumes in comparison to more mature bulls [9]. An added advantage is that unlike frozen-thawed semen, which must undergo a 30 day quarantine period in the European Union, liquid semen can be released for insemination on the day of collection. Despite its advantages, liquid semen has a limited shelf

life and is normally used for only 2.5 to 3 days post collection as a reduction in pregnancy rates has been reported thereafter [10]. In order to combat this reduction in fertility a number of investigative avenues have been exploited with many studies focusing on reducing the metabolic activity of sperm, as sperm survival for extended periods of time has been shown to be inversely related to their metabolic activity [10]. Approaches taken include; altering storage temperatures [11], reducing sperm number in semen diluted in Caprogen [4] as well as N_2 gassing and modifying diluent composition [12], to name but a few.

It is widely acknowledged that reduced storage temperatures and N_2 gassing of media are two primary methods of reducing metabolic activity of sperm. However, storing semen at lower temperatures (ie: 5 ^{o}C) can cause membrane damage and has been reported to cause a rise in intracellular levels of sodium to cytotoxic levels as the activity of the sodium-potassium pump declines [13]. Storage at ambient temperatures (ie: 15 – 20 ^{o}C) avoids the damage sustained by reduced temperatures but it has been postulated that the production of reactive oxygen species is accelerated at higher compared to lower storage temperatures [15, 16]. Protocols to reduce sperm metabolic activity at ambient temperature were devised such as N_2 gassing [14] to dispel oxygen in the media creating an anaerobic environment. INRA96, while marketed for use at 4 ^{o}C in an aerobic environment, has demonstrated sufficient protection capabilities to bull sperm when stored at ambient temperature [6], however, whether the use of N_2 gassing of INRA96 is beneficial remains to be elucidated.

It has previously been reported that increasing the dilution rate, specifically when using an egg-yolk based diluent, was the primary reason for a decline in fertility of liquid semen at lower sperm numbers rather than a direct effect of a lower sperm concentration, as higher

dilution rates adversely affect viability [17, 18]. Excessive dilution can lead to permanent loss of motility, metabolic activity and fertilising capacity [19]. While a larger quantity of egg yolk provides more protection to sperm from the harmful effects of seminal plasma [20] it also provides a substrate for hydrogen peroxide (H_2O_2) production from dead sperm [21], thus contributing to increasing the generation of reactive oxygen species (ROS) which is detrimental to sperm [22]. Previous studies of liquid semen have reported beneficial *in vitro* effects of reducing sperm concentration such as increased sperm viability, reduced oxidative stress [7] as well as a decreased susceptibility to osmotic shock [23]. Shannon, Curson and Rhodes [24] reported no significant reduction in fertility of liquid semen when sperm concentration was reduced from 10 to 1.5 x 10^6 sperm per insemination dose. In contrast, Murphy et al. [4] demonstrated that excessive dilution of sperm had a negative effect on NRR of semen used on Day 2 of storage compared to frozen-thawed semen; however, this did not differ from fertility achieved with liquid semen used on Day 1.

Therefore, using a combination of *in vitro* assessments and a large-scale commercial field trial, the objectives of this study were to assess the effect of N_2 gassing, storage temperature and reducing the sperm concentration in liquid bull semen diluted in INRA96.

2. Materials and Methods

2.1 Experiment 1: Effect of diluting liquid semen in INRA96 with/without nitrogen at 5 or 15 ^{o}C on total and progressive sperm motility.

The aim of this experiment was to assess the effect of N_2 bubbling on total and progressive motility of liquid semen stored for up to 4 days post collection. Semen was collected from Holstein Friesian bulls (n = 5) on three occasions (each occasion/collection was one replicate)

at a commercial AI centre in Ireland. The raw ejaculate was partially diluted in 10 mL pre-warmed INRA96 (IMV Technologies, L'Aigle, France) at 37 ^{o}C and transported in a temperature-regulated cooler box at 18 ^{o}C to the laboratory (1 h transportation). On arrival, the ejaculate was assessed for sperm concentration using a coulter counter (Z Series, Beckman Coulter, Co Clare, Ireland) as well as an initial score of total motility (%) and gross motility on a subjective 5-point scale (1 = twitching/no forward progressive motility; 5 = excellent forward progressive motile sperm) to ensure all semen samples were of a commercial standard (results not shown). Microscopic assessments were conducted by the same technician and initial quality control cut-off values were a total and gross motility of $\geq$70% and a score of $\geq$3, respectively, and any ejaculates failing to meet these criteria were rejected.

The ejaculate was then split into two equal parts and diluted using either N_2 bubbled or non-bubbled INRA96 to achieve a concentration of 5 x 10^6 sperm per 0.25 mL insemination dose. Prior to dilution, N_2 bubbled INRA96 (200 ml) was purged in food fresh Nitrogen gas (BOC, Dublin, Ireland) for 30 min [27] to dispel oxygen from the media and create an anaerobic environment, limiting the metabolic activity of sperm during liquid storage. Semen from each bull was kept separate and ejaculates were split such that each bull was represented equally in each treatment. Semen straws were filled as per routine procedures, placed in an insulated plastic container (to slow temperature shifts) and stored at one of two temperatures, 5 ^{o}C (placed in fridge) and 15 ^{o}C (placed in a temperature regulated box) [4, 10, 14]. Samples from the different treatments were assessed in a randomised sequence to remove bias as a result of sampling order. Total and progressive motility (%); (n = 3 replicates) were subjectively assessed *in vitro* on Days 0, 1, 2, 3 and 4 post-collection (Day 0 = 4 h after collection).

143 2.1.1 Assessment of sperm motility.

144 Sperm motility (total and progressive) in liquid semen was assessed subjectively on Days 0,
145 1, 2, 3 and 4 post semen collection using a phase contrast microscope (CX31; Olympus,
146 Centre Valley, PA, USA) at a magnification of 400 X. A droplet of diluted semen (5 µL) was
147 placed on a pre-warmed glass slide, covered with a pre-warmed coverslip (18 x 18 mm; 37
148 ^{o}C) and assessed by counting a minimum of 100 sperm, over at least five different fields of
149 view, for each treatment on each assessment day. Total motility was expressed as a
150 percentage of the total sperm population (motile and non-motile). A sperm was deemed to
151 display progressive motility if it moved in a linear fashion; progressive motility was
152 expressed as the percentage of motile sperm.

153

154 2.2 Experiment 2a: Effect of storing liquid semen in INRA96 at varying sperm concentrations
155 on total and progressive sperm motility.

156 The aim of this experiment was to assess the effect of bull sperm concentration on total and
157 progressive motility on Day 0, 1 and 2 of storage. Semen was collected from Holstein
158 Friesian bulls (n = 6; 7 collection days; 3 bulls used per collection day resulting in 3 to 4
159 ejaculates/replicates per bull) at a commercial AI centre. The raw ejaculate was collected,
160 partially diluted (with 10 mL of INRA96), transported, assessed and processed as described
161 above. The ejaculate was diluted via a two-step dilution, firstly to achieve a concentration of
162 60 x 10^6 sperm per mL and then to achieve the final concentrations of 3, 4 and 5 x 10^6 sperm
163 per 0.25 mL insemination dose. Semen from each bull was kept separate and ejaculates were
164 split such that each bull was represented equally in each treatment. Semen straws were filled
165 as per routine procedures and stored in a temperature regulated cooler box at 15 ^{o}C. Samples
166 from the different treatments were assessed in a randomised sequence to remove bias as a

result of sampling order. Total and progressive motility; (n = 3 replicates) were assessed *in vitro* as per Experiment 1 on Days 0, 1, 2 and 3 post-collection (Day 0 = 4 h after collection).

2.3 Experiment 2b: The field fertility of liquid semen diluted in INRA96 at 3, 4 or 5 x 10^6 sperm per insemination dose.

The aim of this experiment was to assess the effect of bull sperm concentration on CR following AI with liquid semen. Liquid semen (3, 4 and 5 x 10^6 sperm per 0.25 mL dose) on Days 1 and 2 post collection and frozen-thawed semen (15 x 10^6 sperm per 0.25 mL dose) were used for insemination (supplementary material Figure 1). Semen was collected from Holstein Friesian bulls (n = 6; denoted A-F) at a commercial AI centre during May 2016. There were 7 collection days in total, with 3 bulls used per collection day (total of 21 ejaculates). The raw ejaculate was collected, partially diluted, transported, assessed and processed as described above. Each batch of liquid semen was clearly labelled and distributed for insemination on the day of collection. Liquid semen was stored at ambient temperature [4] and used for up to 2 days post collection on both heifers (n = 391) and multiparous (n = 1,884) dairy cows. Due to logistical constraints, frozen-thawed semen doses were derived from previously collected ejaculates from the same 6 bulls which were processed and frozen using routine procedures (n = 3,467 inseminations consisting of 1,084 heifers and n = 2,383 multiparous dairy cows). Briefly, upon collection of semen samples for cryopreservation, the raw ejaculate was partially diluted in 10 mL of pre-warmed BullXcell (IMV Technologies) at 37 ^{o}C and transported to the laboratory. Semen samples were assessed for volume, sperm concentration and total and progressive motility as described above. Only ejaculates achieving a total and gross motility score of $\geq$70% and $\geq$3 were used for cryopreservation. Following *in vitro* assessments, the semen was fully extended with pre-warmed BullXcell to achieve a concentration of 15 x 10^6 sperm per 0.25 mL insemination dose. Straws were frozen

192 to -140 °C as follows: -5 °C per min from +4 °C to -10 °C, -40 °C per min from -10 °C to -100
193 °C and thereafter -20 °C per min from -100 °C to -140 °C in a programmable freezer
194 (Digitcool; IMV Technologies), followed by submersion and storage in liquid nitrogen at -
195 196 °C until use.

196 Inseminations (liquid and frozen-thawed semen) were carried out in May 2016
197 (coinciding with the peak dairy breeding season) in Irish dairy herds (n = 750). The majority
198 of inseminations were in Holstein Friesian cows (n = 5,476) but small numbers of cows of
199 other breeds were represented: Jersey (n = 158), Montbeliarde (n = 40), Norwegian Red (n =
200 34) and other (n = 34; includes Normande, Rotbunte, Swedish Red, Danish Red and Red
201 Poll). Technicians (n = 61) were grouped into geographical areas and treatments were rotated
202 on each collection day to ensure that technicians, who were blinded to treatments, received
203 different diluent treatments from each of three bulls on each day (supplementary material
204 Figure 2). For each insemination the AI technician recorded the bull code, cow tag number
205 and the straw code on a handheld electronic device.

206 Cow characteristics such as parity, days in milk (DIM) and fertility sub-index were
207 also assessed. Fertility sub index, a key component of the Economic Breeding Index (EBI)
208 comprises ~35% of the total EBI and is based on calving interval and cow survival [25]. It
209 was set up to combat a decline in reproductive performance by providing farmers with a
210 profit index enabling the selection of elite sires to breed replacement heifers with increased
211 milk yield, reproductive performance and improved health traits [26].

212

213 2.3.1 Capturing of calving rate data

214 Calving rate data were captured using the Irish Cattle Breeding Federation (ICBF; Bandon,
215 Co Cork) database by cross-referencing the technician name with the bull code and semen
216 type used on each date within the trial period. Obvious errors were extracted from the dataset

and data were then interrogated to remove animals based on the following criteria: cows which were not at first AI, cows which received two inseminations from two different bulls or sperm concentration treatments, or cows which were not of a dairy breed. However, if a cow received two inseminations from the same bull with the same sperm concentration treatment within five days of each other, the record was kept and the second date was assumed to be correct. Post editing, a total of 5,742 inseminations remained. Calving rate was measured using a cut-off value of 275 and 290 days from date of insemination to calving date.

2.4 Statistical analysis

Data from Experiments 1 and 2a were examined for normality of distribution, homogeneity of variance and analysed using the general linear model (GLM) repeated-measures procedure with a compound symmetry covariance structure in Statistical Package for Social Science (SPSS, Version 22.0; IBM, Chicago, USA). The final model for Experiment 1 included the main effects of N_2 bubbling, day, temperature and their interaction. The final model for Experiment 2a included the main effects of sperm concentration treatment, day and their interaction. In Experiment 2b, CR data were compared using Pearson's chi-squared procedures in SPSS. Data were cross checked using an analysis of variance (ANOVA) model. The dependent variable in the analysis was CR (1 = calved, 0 = not calved). In addition, using a general linear model for binomial data, CR data and correlations were investigated with a number of fixed effects, namely sperm concentration, bull, parity number, cow breed, cow fertility sub-index, days in milk (DIM), herd and technician. Each fixed effect was assessed for an interaction with sperm concentration. All post-hoc tests were carried out using Bonferroni test and results are reported as the mean $\pm$ the standard error of the mean (s.e.m) in Experiments 1 and 2a and the estimated marginal means in Experiment

2b, to adjust for the imbalance between the number of inseminations in each comparison/treatment. Data were considered to differ significantly at $P < 0.05$.

3. Results

3.1 Experiment 1: Effect of diluting liquid semen in INRA96 with/without nitrogen at 5 or 15 ^{o}C on total and progressive sperm motility.

There was no effect of N_2 bubbling on total and progressive motility of liquid semen (Figure 1). However, there was an effect of temperature, day and a day x treatment interaction on total and progressive motility ($P < 0.01$). As expected, total and progressive motility declined with increased duration of storage ($P < 0.01$). Semen held at 15 ^{o}C had a higher total and progressive motility score throughout the duration of storage compared to semen held at 5 ^{o}C regardless of N_2 treatment (N_2 bubbled or non-bubbled INRA96; $P < 0.01$). There was no effect of bull, bull x day and or bull x temperature interaction on total and progressive motility.

3.2 Experiment 2a: Effect of storing liquid semen in INRA96 at varying sperm concentrations on total and progressive sperm motility.

Although total and progressive motility declined linearly with increased duration of storage ($P < 0.01$), there was no effect of sperm concentration on both total and progressive motility. Semen diluted to a concentration of 3, 4 and 5 x 10^6 sperm per dose maintained acceptable total and progressive motility scores throughout the duration of storage with a decline in total (81.8 to 74.2, 81.6 to 73.3 and 81.9 to 75.3%, respectively) and progressive motility (83.3 to 74.4, 84.3 to 74.4 and 84.2 to 75.6%, respectively) on Days 0, 1 and 2 of storage. There was also no sperm concentration by day interaction.

267 3.3 Experiment 2b: The field fertility of liquid semen diluted in INRA96 at 3, 4 or 5 x 10^6
268 sperm per insemination dose.

269 Overall, inseminations with liquid semen on Day 1 post collection resulted in similar CR
270 (52.1%) in comparison to frozen-thawed semen (55.2%). Insemination with liquid semen on
271 Day 2 of storage resulted in a lower CR (45.6%; $P < 0.01$) compared to semen used on Day 1
272 or frozen-thawed semen (52.1 and 55.2%, respectively; $P < 0.01$). There was an effect of
273 sperm concentration on CR following AI as liquid semen diluted to 3 or 4 x 10^6 sperm per
274 dose resulted in a lower CR on Day 2 of storage (41.1 and 44.7%, respectively; $P < 0.05$) in
275 comparison to frozen-thawed semen ($P < 0.05$) but did not differ to semen diluted to 5 x 10^6
276 on Day 2 of storage (Figure 2). There was no effect of bull on CR with the average CR for
277 bulls used in the trial varying between 50.7 and 54.9 %. There was a bull x sperm
278 concentration interaction ($P < 0.05$), as Bulls D and E in the 3 x 10^6 treatment had a lower CR
279 (47.6 and 43.7%, respectively) in comparison to frozen-thawed semen (58.3 and 61.7%,
280 respectively). There was a bull x day interaction as Bulls B and E had a lower CR on Days 1
281 and 2 (Bull B; 42.2 and 42.3% vs Bull E; 48.8 and 40.2% for Day 1 and 2, respectively) in
282 comparison to frozen-thawed semen (58.7 and 61.7%, respectively; $P < 0.05$) while Bulls C
283 and D had a reduced CR on Day 2 (44.0 and 47.5%, respectively), in comparison to frozen-
284 thawed semen (54.8 and 58.3%, respectively; $P < 0.05$).

285 There was an effect of parity, cow fertility sub-index and DIM on CR ($P < 0.01$).
286 Maiden heifers had a higher CR (57.9%) than primiparous (52.7%; $P < 0.05$) and multiparous
287 dairy cows (50.6%; $P < 0.01$). Cows with a fertility sub-index of greater than €110 recorded a
288 higher CR (61.0%) in comparison to cows with a fertility sub-index of <€50, €50-70, €70-90
289 and €90-110 at 45.6, 51.0, 53.8 and 55.4%, respectively. Cows greater than 60 DIM at the
290 time of AI recorded a higher CR (55.4%) than cows with a DIM of <20, 20-40 and 40-60
291 (CR of 22.7, 36.3 and 41.2%, respectively). As expected, CR varied between individual herds

292 and technicians, for herds and technicians with greater than 40 and 45 recorded
293 inseminations, respectively ($P < 0.01$). There was no effect of breed, or a breed, cow fertility
294 sub-index, DIM, herd or technician by sperm concentration interaction.
295
296 **4. Discussion**
297 The main findings of this study were (i) storage of bull semen in INRA96 at 15 ^{o}C is superior
298 to semen stored at 5 ^{o}C as assessed *in vitro* (ii) N_2 bubbling of INRA96 or reduced sperm
299 concentration (within the range 10^6 sperm per dose) had no effect on total and progressive
300 motility *in vitro*, and (iii) insemination with liquid semen stored at 3 and 4 x 10^6 sperm per
301 dose resulted in a reduced CR following 2 days of storage in comparison with frozen-thawed
302 semen but did not differ from semen diluted to 5 x 10^6 sperm per dose on Day 2 of storage.
303 As each treatment was prepared from the same ejaculate, any potential cofounding effects of
304 day of collection were removed, thus providing clear and reliable *in vitro* and *in vivo* data on
305 the use of liquid bull semen.
306
307 It has previously been reported that sperm are quite versatile in relation to sperm quality
308 between storage temperatures of 5 – 22 ^{o}C as they were found to retained acceptable *in vitro*
309 standards [4], with semen stored at 15 ^{o}C having greater motility compared to semen stored at
310 5, 22, 32 ^{o}C or fluctuating temperatures between 5-15, 5-22 and 5-32 ^{o}C. This is in agreement
311 with the results of the current study as although semen held at both a constant 15 or 5 ^{o}C
312 recorded acceptable total and progressive motility values up to 4 days of storage, storing
313 semen at 15 ^{o}C resulted in better total and progressive motility throughout the duration of
314 storage compared to semen stored at 5 ^{o}C. In the current study N_2 bubbling had no effect on
315 sperm motility regardless of storage temperature. This is in agreement with Krzyzosiak et al.
316 [27] who reported no significant difference in the percentage of motile bull sperm up to Day

3 of storage or on the *in vitro* fertility of sperm stored under aerobic, N_2 gassed and anaerobic conditions (N_2 gassed and placed in an anaerobic chamber overnight with 5% Hydrogen and 95% N_2). Although not assessed in the current study, plasma membrane integrity deteriorated quicker when stored in aerobic conditions compared to anaerobic or N_2 gassed conditions [27], however, motility on Day 7 was significantly lower after storage under anaerobic conditions compared to aerobic or N_2 gassed storage conditions [27]. Therefore, it may be possible that diluents provides sufficient support for sperm motility over prolonged periods (up to 3 days of storage) regardless of the oxygenated state of the media and that the benefits of N_2 gassing are not observed by assessing motility alone.

As expected, in the current study, total and progressive motility declined linearly in all treatments with increased duration of storage regardless of storage temperature, N_2 gassing or sperm concentration; however, although all sperm concentration treatments maintained acceptable total and progressive motility on Day 2 of storage with scores above 74% respectively, semen diluted to 3 and 4 x 10^6 sperm per dose resulted in a reduced CR on Day 2 of storage (41.1 and 44.7%, respectively) compared to frozen-thawed semen (55.2%). A study conducted by Vishwanath and Shannon [16] reported that bull sperm have gradual decreasing motility scores for up to 4 weeks when stored in Caprogen diluent; however, there was a sharp decline in NRR after 3-5 days (69.9 ± 1.2%) compared to after 10 days (41.5 ± 3.7%). This suggests that additional factors relating to fertility other than sperm motility are essential in achieving high pregnancy rates.

Murphy et al. [4] previously reported that some bulls are more susceptible to sperm aging resulting in a decrease in their fertility due to the cumulative generation of reactive oxygen species with increased duration of storage. However, the results of the current study would

suggest that some bulls may be more suitable for use in frozen-thawed semen rather than liquid semen programs regardless of the duration of storage. Three bulls in particular performed relatively poorly, in relation to CR, following AI on Day 1 and 2 of storage of liquid semen compared to frozen–thawed semen with these bulls combined having an average CR of 45.9% and 42.4% on Day 1 and 2, respectively, compared to 58.4% for frozen-thawed semen. Notwithstanding the limitations of the relatively modest number of inseminations per bull [28] a possible explanation for poor fertility performance of liquid semen may be due to the inability of the sperm to adapt to temperature variation associated with storage of liquid semen at ambient temperature. A previous study by our group found that semen stored at ambient temperatures in unregulated temperature control boxes in the trunk of a car (similar storage conditions to liquid semen in Ireland) where subjected to day to night time temperature variations with minimum and maximum temperature values of 6.4 and 27.9 ^{o}C, respectively, [4]. Although Murphy et al. [6] reported that storing diluted semen at constant or fluctuating temperatures between 4 – 18 ^{o}C had no impact on motility when stored in INRA96, unpublished data by our group shows that while sperm are quite versatile in terms of storage temperature, fluctuating temperatures between 4 – 28 ^{o}C (night-time to daytime over 4 days) resulted in a significant loss of motility (unpublished). Exposure to such daytime/night-time temperature fluctuations may result in a decline in membrane integrity as a consequence of morphological membrane changes which are consistent with the lipid phase transition [29], thus resulting in a reduction in sperm quality and fertility [30]. Therefore, further investigation should be undertaken with a strict temperature regulation regime in place so that liquid semen is maintained at a constant temperature.

In the current study, CR of liquid semen stored at ambient temperature and diluted in INRA96 to 3 and 4 x 10^{6} sperm per dose on Day 2 of storage decreased significantly

367 compared to frozen-thawed semen but did not differ from semen diluted to 5 x 10^6 sperm per
368 dose after 2 days of storage. Additionally, CR of semen diluted to 3, 4 or 5 x 10^6 sperm per
369 dose on Day 1 of storage did not differ from frozen-thawed semen. This supports the findings
370 of Murphy et al. [4] as semen diluted in Caprogen had a reduced NRR on Day 2 of storage at
371 lower concentrations compared to frozen-thawed semen. However, in the current study, the
372 overall CR of liquid semen following AI on Day 1 of storage was comparable to the previous
373 study reported by Murphy et al. [6] who compared semen diluents at the higher concentration
374 of 5 x 10^6 sperm per dose. Consequently, it could be recommended to increase sperm
375 numbers for targeted use on subsequent days after collection; namely 4 and 5 x 10^6 sperm per
376 dose for insemination on Day 1 and 2 post collection, respectively. This would ensure that the
377 number of insemination doses per ejaculate is maximised, thus resulting in an increase in the
378 use of individual sires. In contrast to the results of this study, previous studies have shown
379 that similar conception rates can be achieved with liquid and frozen-thawed semen [31] and
380 that NRRs of liquid and frozen-thawed semen diluted to 2.5 x 10^6 and 20 x 10^6 sperm,
381 respectively, do not differ up to Day 2 of storage [32]. However, over-compensation of sperm
382 numbers typically occurs in the preparation of frozen-thawed semen, resulting in a sperm
383 concentration which considerably exceeds the number of sperm necessary for maximum
384 fertility, thus, masking the 'true fertility' potential of a bull [33].
385
386 It is well established that cow characteristics such as parity, fertility sub-index and DIM play
387 a role in fertility [4, 6]. Cow fertility declines with increased age as stresses associated with
388 calving, lactation and clinical abnormalities at parturition or postpartum have a negative
389 effect on fertility [34, 35]. Previous studies have found that heifers had a higher NRR
390 compared to multiparous dairy cows [36, 37]. The results of the current study show that,
391 while numerically greater, CR of heifers did not differ to primiparous dairy cows but was

392 ~13% higher than multiparous dairy cows with a parity of greater than 5. Furthermore, there
393 was no treatment by parity interaction highlighting that no particular semen type has an
394 advantage in negating the effects of parity. In the current study, a positive linear relationship
395 between fertility sub-index and CR was observed with animals in the highest fertility sub-
396 index (>€110) having a higher CR than any other sub-index. Therefore, the results clearly
397 highlight the importance of the EBI and its contribution to herd fertility. In this study, the
398 number of DIM affected CR with cows of greater than 60 DIM having a higher CR than
399 those of less than 60 DIM. Therefore, this study demonstrates that increasing the number of
400 DIM increases fertility; consequently, allowing a DIM of 60 days can be beneficial to the
401 overall production of a dairy herd. However, in the case of late calving cows, some
402 confidence can also be taken from inseminating these animals at a shortened DIM interval,
403 with reasonable success, in order to bring forward the calving date of this cohort of cows.
404

405 **5. Conclusion**

406 In conclusion, storing semen at 15 ^{o}C resulted in superior total and progressive motility
407 values compared to semen stored at 5 ^{o}C, while N_2 gassing and sperm concentration (3, 4 or 5
408 x 10^6 sperm per dose) had no effect on sperm motility. On Day 1 of storage there was no
409 difference in CR between liquid and frozen-thawed semen; however, on Day 2 of storage
410 insemination with 3 or 4 x 10^6 sperm per dose resulted in a lower CR in comparison to
411 frozen-thawed semen but did not differ from 5 x 10^6 sperm per dose. Thus, given that
412 reducing sperm concentration per dose results in an increased number of doses per ejaculate,
413 therefore increasing the utilisation of superior sires, it may be justifiable to increase sperm
414 numbers for targeted use on subsequent days after collection. Furthermore, additional
415 consideration should be given to the storage temperature of liquid semen with an aim to
416 storing liquid semen at a constant temperature.

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422 **Figure captions**

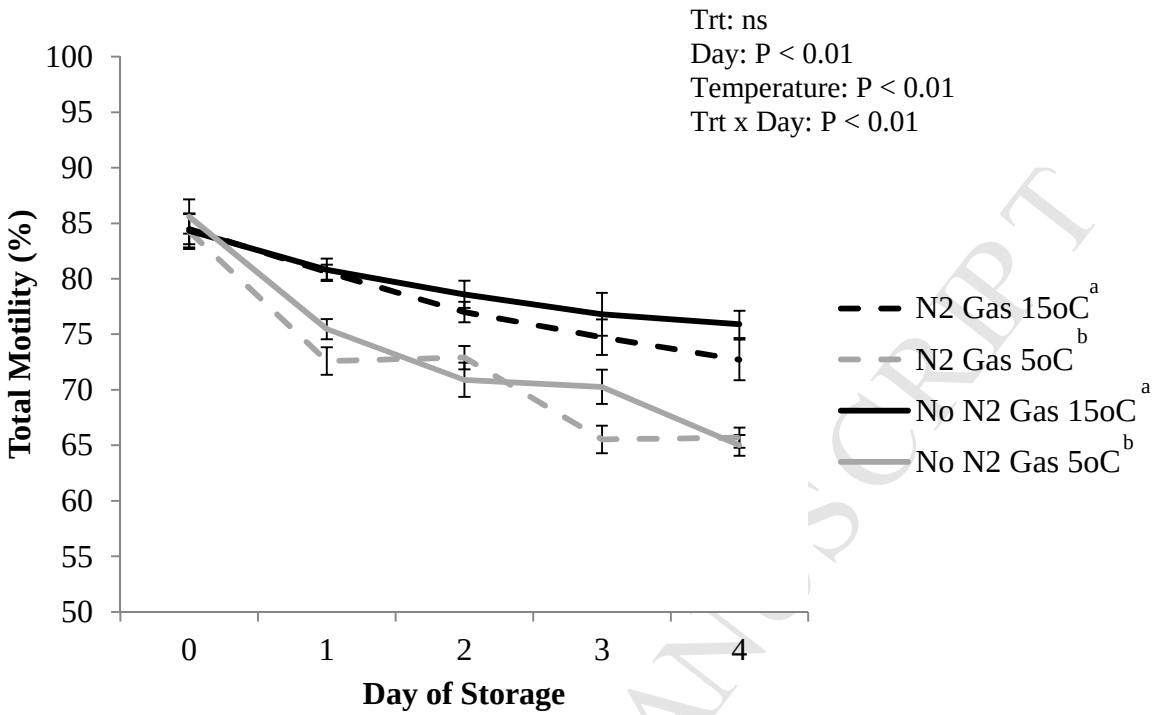
423 **Figure 1:** The effect of nitrogen bubbling on total (upper panel) and progressive (lower
424 panel) motility of liquid semen on Days 0, 1, 2, 3 and 4 post collection (n = 5 bulls; 3
425 ejaculates/replicates per bull; Experiment 1). Vertical bars represent ± s.e.m. [ab]Treatments
426 with different superscripts differ significantly (P < 0.01). Trt = treatment, ns = non-
427 significant.

428

429 **Figure 2:** The effect of sperm concentration and day of storage on calving rate in dairy cows
430 and heifers (n = 6 bulls; 3 to 4 ejaculates/replicates per bull; Experiment 2b). [ab]Differing
431 superscripts differ significantly between treatments (P < 0.01). Vertical bars represent 95%
432 confidence intervals. n = the total number of inseminations per treatment per day.

433 Murphy et al. Figure 1.

434



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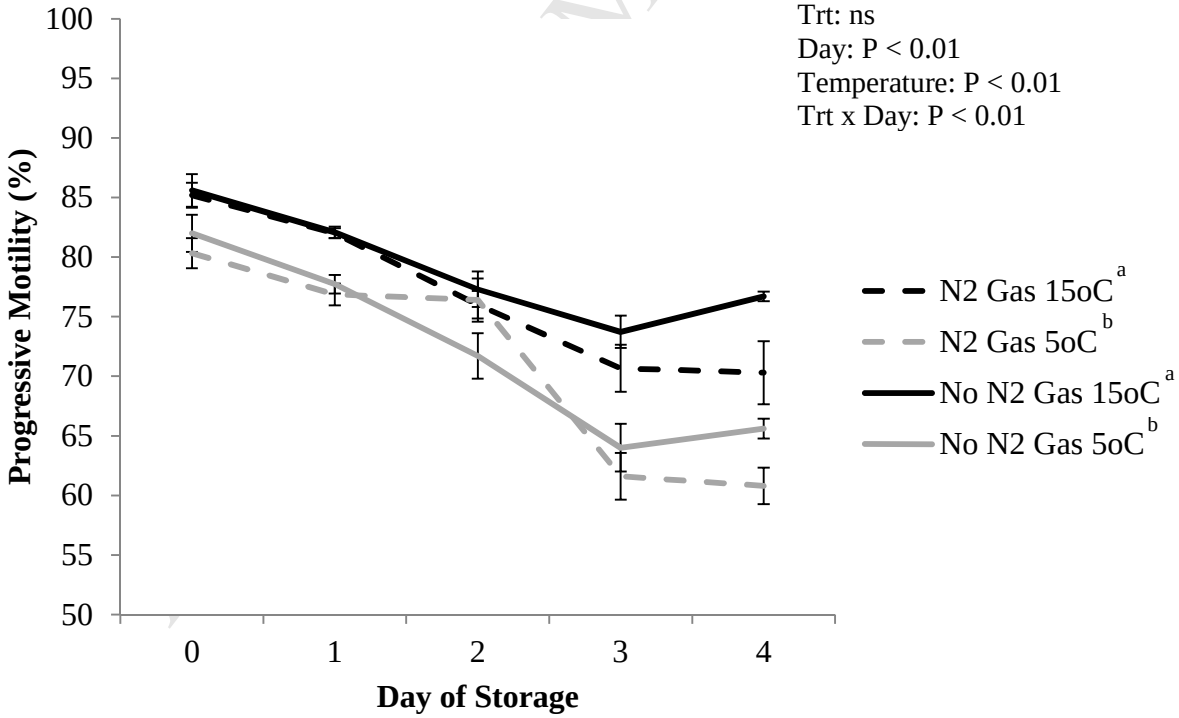

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438 Murphy et al. Figure 2.

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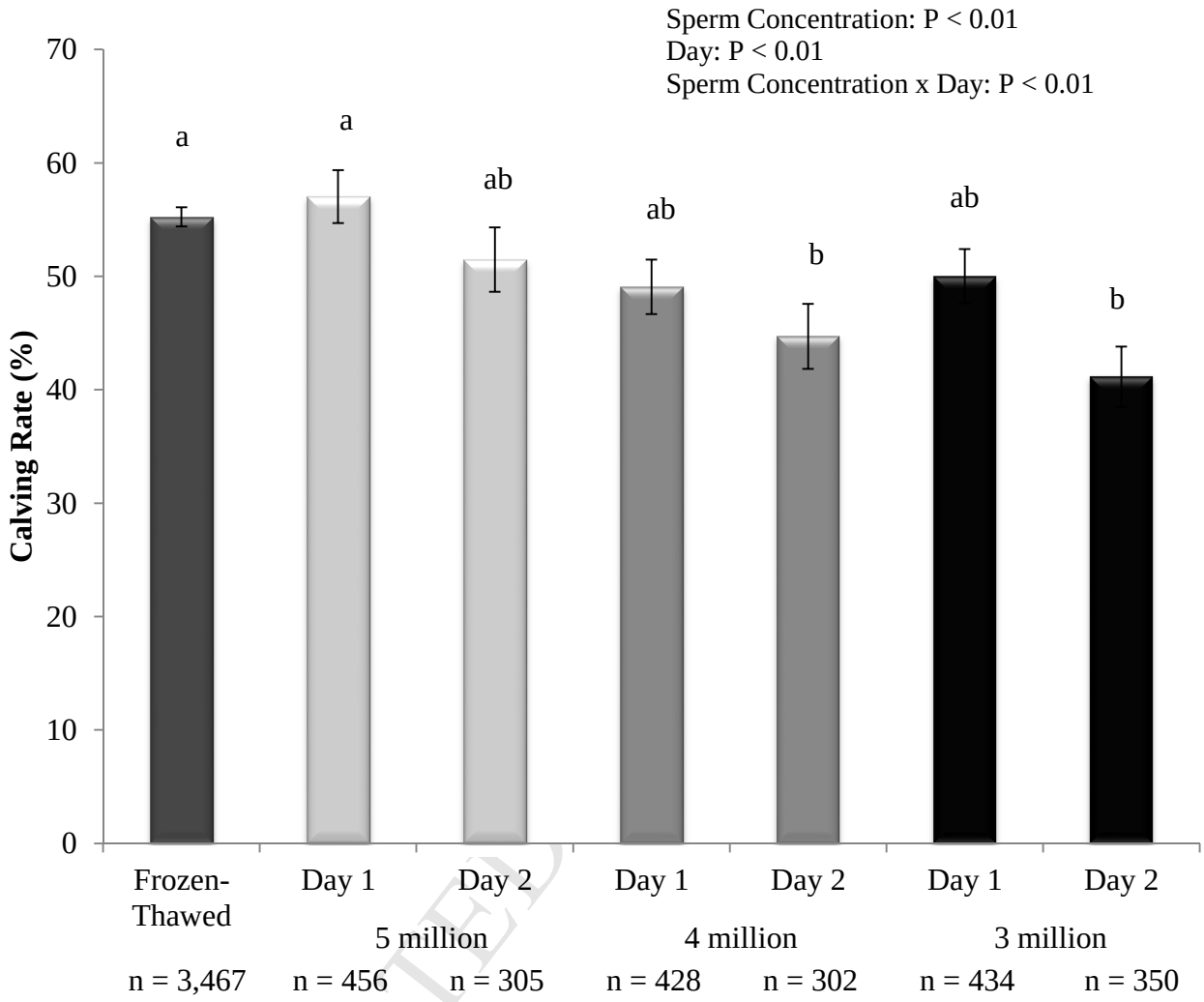

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Highlight for review

- Storage of bull semen in INRA96 at 15 ^{o}C is superior to semen stored at 4 ^{o}C, as assessed *in vitro*.
- N_2 bubbling of INRA96 or reduced sperm concentration (within the range 3 to 5 x 10^6 sperm per dose) had no effect on total and progressive motility.
- Insemination with liquid semen stored at 3 and 4 x 10^6 sperm per dose in INRA96 resulted in a reduced calving rate following 2 days of storage in comparison with frozen-thawed semen.